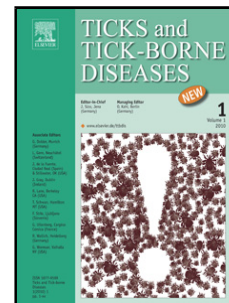


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## Relapsing fever causative agent in southern Iran is a closely related species to East African borreliae

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**Highlights** We identified relapsing fever patients from Hormozgan Province, South Iran, where *O. erraticus* ticks predominate.

- Molecular characterization of the causative spirochete revealed the greatest identity with East African borreliae, *Borrelia recurrentis*, *Borrelia duttonii*, and *Borrelia microti* from Iran.
- The more discriminatory IGS separated this *Borrelia* species from *B. microti* and indeed other relapsing fever borreliae.
- The spirochete identified in our study might represent an ecotype-adapted strain of East African *B. duttonii*.

### Abstract

We obtained two blood samples from relapsing fever patients residing in Jask County, Hormozgan Province, southern Iran in 2013. Sequencing of a partial fragment of *glpQ* from two samples, and further characterization of one of them by analyzing *flaB* gene, and 16S-23S spacer (IGS) revealed the greatest sequence identity with East African borreliae, *Borrelia recurrentis*, and *Borrelia duttonii*, and *Borrelia microti* from Iran. Phylogenetic analyses of *glpQ*, *flaB*, and concatenated sequences (*glpQ*, *flaB*, and IGS) clustered these sequences amongst East African Relapsing fever borreliae and *B. microti* from Iran. However, the more discriminatory IGS disclosed a unique 8-bp signature (CAGCCTAA) separating these from *B. microti* and indeed other relapsing fever borreliae. In southern Iran, relapsing fever cases are mostly from localities in which *O. erraticus* ticks, the notorious vector of *B. microti*, prevail. There are chances that this argasid tick serves as a host and vector of several closely related species or ecotypes including the one we identified in the present study. The distribution of this *Borrelia* species remains to be elucidated, but it is assumed to be endemic to lowland areas of the Hormozgan Province, as well as Sistan va Baluchistan in the southeast and South Khorasan (in Persian: Khorasan-e Jonobi) in the east of Iran.

**Keywords:** Relapsing fever; *Borrelia duttonii*; *Borrelia recurrentis*; Molecular characterization; East Africa; Southern Iran.

## 1. Introduction

Soft tick-borne relapsing fever (STBRF) is an endemic disease in Iran, with more than 140 cases annually throughout the country during 1997-2006 (Masoumi Asl et al., 2009). In Iran, four *Borrelia* species, *Borrelia persica*, *Borrelia microti*, *Borrelia baltazardi*, and *Borrelia latyschewii* are known causes of relapsing fever. In the western, northwest, and foothill regions of the Alborz Mountains stretching to east in Khorasan Razavi Province, the argasid tick *Ornithodoros tholozani* is commonplace in animal shelters and adjacent human dwellings accounting for most STBRF cases attributed to *B. persica* infection (Karimi et al., 1979, Karimi, 1981, Masoumi Asl et al., 2009). However, in central and western Iran *Borrelia microti*-infected *Ornithodoros erraticus* ticks coexist with *O. tholozani*, and in southern Iran, this tick predominates in the absence of *O. tholozani* (Janbakhsh and Ardelan, 1977, Karimi, 1981). Two other *Borrelia* species have been identified in Iran, *B. latyschewii*, isolated from *Ornithodoros tartakowskyi* ticks in the southeast (Piazak et al., 2000) and *B. baltazardi* that was described in a febrile patient with thrombocytopenic purpura in Ardebil, an endemic area for STBRF *B. persica* in northwest of the country (Karimi et al., 1979, Karimi, 1981). *Borrelia baltazardi* was discriminated from *B. persica* using electron microscopy (Karimi et al., 1979) and experimental pathogenicity in animal models including adult Guinea pigs, rats, mice, and newborn rabbits (Karimi et al., 1979, Karimi, 1981, Assous and Wilamowski, 2009). This spirochete was only isolated once and attempts to maintain it in laboratory animals failed; no tick vector has been identified for this species to date (Karimi et al., 1979, Karimi, 1981). Molecular characterization of *B. persica* from Iran has been reported (Ras et al., 1996, Shirani et al., 2016), but the characterization of other Iranian borreliae remains scarce. Phylogenetic analysis using concatenated sequences of 16S rRNA, *flaB*, and *glpQ* grouped Iranian *B. microti* alongside African species, *B. duttonii*, *B. recurrentis*, and *B. crocidurae*, and distinct from *B. persica*, the commonly established cause of STBRF in Iran (Naddaf et al., 2012). Similarly, 16S-23S intragenic sequence (IGS) analysis conducted *in situ* using blood from relapsing fever patients in Hormozgan Province, southern Iran showed greatest similarity with East African relapsing fever

*borreliæ*, *B. recurrentis* and *B. duttonii*, but was surprisingly distinct from *B. microti* transmitted by *O. erraticus* ticks, previously believed to be the only soft tick species in this region (Naddaf et al., 2015). In this study, we further characterize the causative agents of relapsing fever amongst patients from Hormozgan Province, southern Iran by sequencing partial fragments of *glpQ*, *flaB*, and IGS.

## **2. Materials and Methods**

Two blood samples from relapsing fever patients residing in Jask County, Hormozgan Province, south of Iran were obtained during 2013. The patients were farmers and had no history of travel to other areas of the country or abroad. Animal inoculation or *in-vitro* culture of the samples was not possible as they had been kept in -20°C for an extended period. The samples were reidentified; the human subject study had been approved by the ethical committee of Pasteur Institute of Iran (Project No. 794). These patients presented with fever, headache, and fatigue and were treated with 500 mg tetracycline orally every 6 hours for ten days and became afebrile. Neither of the treated patients developed Jarisch-Herxheimer reaction or any other adverse consequences.

### **DNA extraction and PCR**

DNA was extracted from blood samples by using the Miniprep DNA kit (QIAGEN, Hilden, Germany). Partial sequences, of the *glpQ*, and IGS were amplified with reagents and PCR conditions as previously described (Halperin et al., 2006, Cutler et al., 2010). We also amplified a partial sequence of the flagellin gene (*flaB*) gene by a nested PCR using the outer primers utilized by Assous et al. (Assous et al., 2006) and the inner primers NBOR-F 5' tgggcatagaattaatcgtg 3', and NBor-R 5' tacttggtgagcaccctcac 3' designed in this study. To prevent cross-contamination, we performed DNA extraction and amplification in separate laboratories and included negative controls in each assay.

### **Phylogenetic analysis**

The sequences generated in the present study were aligned with appropriate sequences from GenBank database. The phylogenetic trees were constructed by using the Jukes-Cantor option of the neighbor-joining method in a complete deletion procedure using MEGA6 software

(Tamura et al., 2013). The robustness of the topologies was estimated through 2,000 bootstrap replications.

### **GenBank submission**

The sequences from this study were submitted to the GenBank under the accession numbers KX683864 and KX683865 for *glpQ*, KX683866 for *flaB*, KX683867 for IGS.

## **3. Results**

### ***glpQ* sequence**

The two 651 and 647-bp *glpQ* sequences of relapsing fever patients were identical over 640 bp. In BLAST sequence analysis, these sequences were identical to those of *B. recurrentis* strains A1, Bek1, and 107 (acc. Nos. KJ003842, CP000993, and AF247152), and clone 2015 (acc. No. KT764112). They showed 99% identity with *B. microti* IR-1, and *B. duttonii* Ly (acc. Nos. JF825473, and CP000976); there was only one nucleotide difference (C > T) at the nucleotide position 261307 corresponding to *B. duttonii* Ly *glpQ* sequence (Fig. 1A). Resequencing of the *glpQ* sequences of the isolates and *B. microti* IR-1 confirmed the presence of this SNP.

### ***flaB* sequence**

We succeeded to amplify a 484-bp fragment from only one specimen (IR-J3) by using nested primers. In BLAST analysis, the *flaB* sequence gave complete identity (100%) with *B. duttonii* strains 1120K3, CR2A, and 406K (acc. Nos. GU357617, GU357618, and D82859), and *B. crociduræ* (acc. No. X75204). It showed 99% identity, equivalent to a single nucleotide difference (C > A) with *B. microti* IR-1, and *B. duttonii* Ly at the position 160681 corresponding to *B. duttonii* Ly *flaB* sequence (acc. No. CP000976), and *B. recurrentis* A1 (A > G) at the position 160700 corresponding to *B. recurrentis* A1 (acc. No. CP000993) (Fig. 1B).

### **IGS sequence**

We amplified a 561-bp IGS from one specimen (IR-J3). This sequence was identical to the IGS type (acc. No. KM271987) and differed by four nucleotides from another one (acc. No. KM271988) previously reported from Jask and Rodan counties in the same area (Naddaf et al., 2015). In multiple sequence alignment, the IGS sequences from south Iran (marked with an asterisk) demonstrated various SNPs, however, a sequential indels and substitutions along a 9-bp fragment corresponding to the nucleotides 456599-456608 in *B. duttonii* Ly (Acc. No.

CP000976) and a single SNP at the position 456529 discriminated these sequences (Fig. 1C). In multiple sequence alignments (with a gap opening penalty 15, and gap extension penalty 6.66, the template format for alignment in MEGA6) these nucleotide changes appeared as a unique 8-bp signature (CAGCCTAA), which was absent in all aligned relapsing fever borreliae including the Iranian *B. microti* IR-1 (Fig. 1D).

### Phylogenetic analysis

Phylogenetic analyses of *glpQ*, *flaB*, and concatenated sequences (*glpQ*, *flaB*, and IGS) clustered these sequences amongst East African relapsing fever borreliae and *B. microti* from Iran (Fig. 2, A, B and C). However, the more discriminatory IGS separated the Iranian sequence types (marked with an asterisk) obtained in the present study (acc.No. KX683866), and the previous one (Naddaf et al., 2015) (acc.Nos. KM271897 and KM271988) from *B. microti* and indeed other relapsing fever borreliae (Fig. 2D).

### 4. Discussion

In recent years, reported numbers of relapsing fever patients in Iran have decreased sharply; being partially attributed to the improvement of housing in rural areas. However, removal of relapsing fever from mandatory reporting to Ministry of Health and Medical Education (MHME) might have contributed to the disease being substantially underreported. Indeed, neither of the relapsing fever patients included in this study was registered in MHME database. Previously, we reported novel *Borrelia* IGS types detected *in situ* from relapsing fever patients in Hormozgan Province, which demonstrated the greatest sequence identity with East African borreliae and *B. microti* from Iran, though formed a distinct group separate from these species (Naddaf et al., 2015). Previously, attempts to characterize other genes had failed. In the present study, the *Borrelia glpQ*, *flaB*, and IGS sequences from other relapsing fever patients residing in the same area further corroborated our earlier observations, demonstrating greatest sequence similarity with East African *Borrelia* species. Our *glpQ* and *flaB* sequences firmly clustered sequences from the Iranian cases amongst strains of East African borreliae, *B. recurrentis*, and *B. duttonii*. As *B. recurrentis* is believed to be a louse-borne adapted strain of *B. duttonii* with 20.4% degradation in genome size (Lescot et al., 2008), it is unsurprising that both species showed almost equivalent sequence identity with *glpQ* and *flaB* analysis. Both *B.*

*microti* and the sequences described herein from Iran fall within this clade. However, the more discriminatory IGS analysis both from this study and previously (Naddaf et al., 2015) revealed a clear separation by possession of a unique 8-bp signature, absent in all closely related East African borreliæ and Iranian *B. microti* sequences. Figure 3 illustrates the geographical distribution of Iranian relapsing fever borreliæ and *B. duttonii* and *B. recurrentis* in the East Africa. In the south Iran, relapsing fever cases are mostly from localities in which *O. erraticus* ticks, the notorious vector of *B. microti*, are reported from rodent burrows (Janbakhsh and Ardelan, 1977). No exhaustive study on the identity of the argasid tick vector, particularly the species residing in rodents burrows is available, and the data on the molecular identity of *B. microti* is scarce and confined to one strain from the center of Iran (Naddaf et al., 2012). There are chances that in the south of Iran *O. erraticus* ticks or similar species serve as a host and vector of closely related *Borrelia* species or ecotypes including the one we identified in the present study. The distribution of this *Borrelia* species is not yet known in Iran but detection will now be possible given its unique molecular signature; its geographical range requires further investigation, but it is assumed to be distributed in lowland areas of the Hormozgan Province, as well as Sistan va Baluchistan in the southeast and South Khorasan (in Persian: Khorasan-e Jonobi) in the east.

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## Figures legends

**Fig. 1.** Multiple sequence alignment of *glpQ* (A) and *flaB* (B) genes and IGS (C and D) of East African and Iranian borreliae. IGS (C) without gap opening and gap extension penalties, and (D) with the gap opening penalty 15, and gap extension penalty 6.66 (the template format for alignment in MEGA6). The IGS sequences from South Iran are marked with an asterisk (\*).

**Fig. 2.** Phylogenetic analyses of *glpQ* (A), *flaB* (B), IGS (D), and concatenated (C) sequences of relapsing fever borreliae. The sequences generated in the present study are in bold, and the IGS from South Iran are marked with an asterisk (\*). The accession number for each sequence is reflected in parentheses. The scale bars represent genetic distances in nucleotide substitutions, 0.01= 1% divergence. The numbers above the branches indicate the percentage of bootstrap samplings percentages, the values below 80 were deleted.



**Fig. 3.** Geographical distribution of Iranian relapsing fever borreliae and East African *B. duttonii* and *B. recurrentis*.

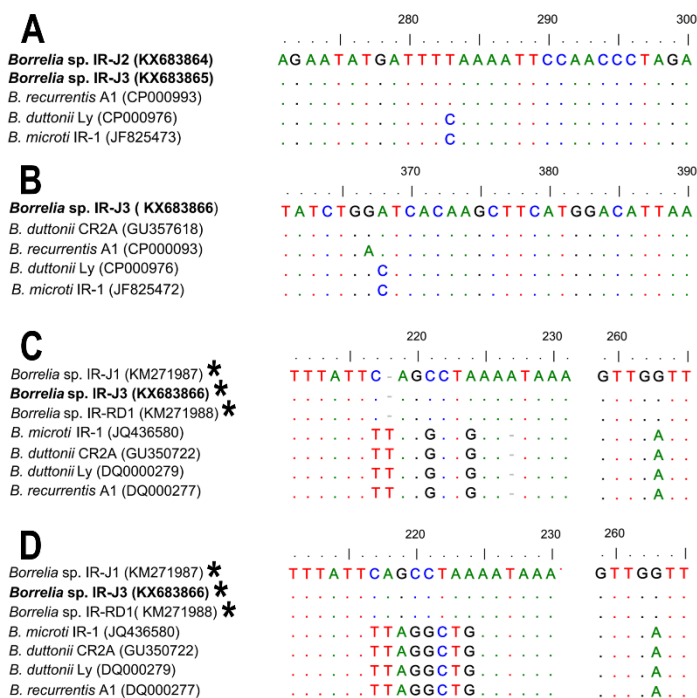
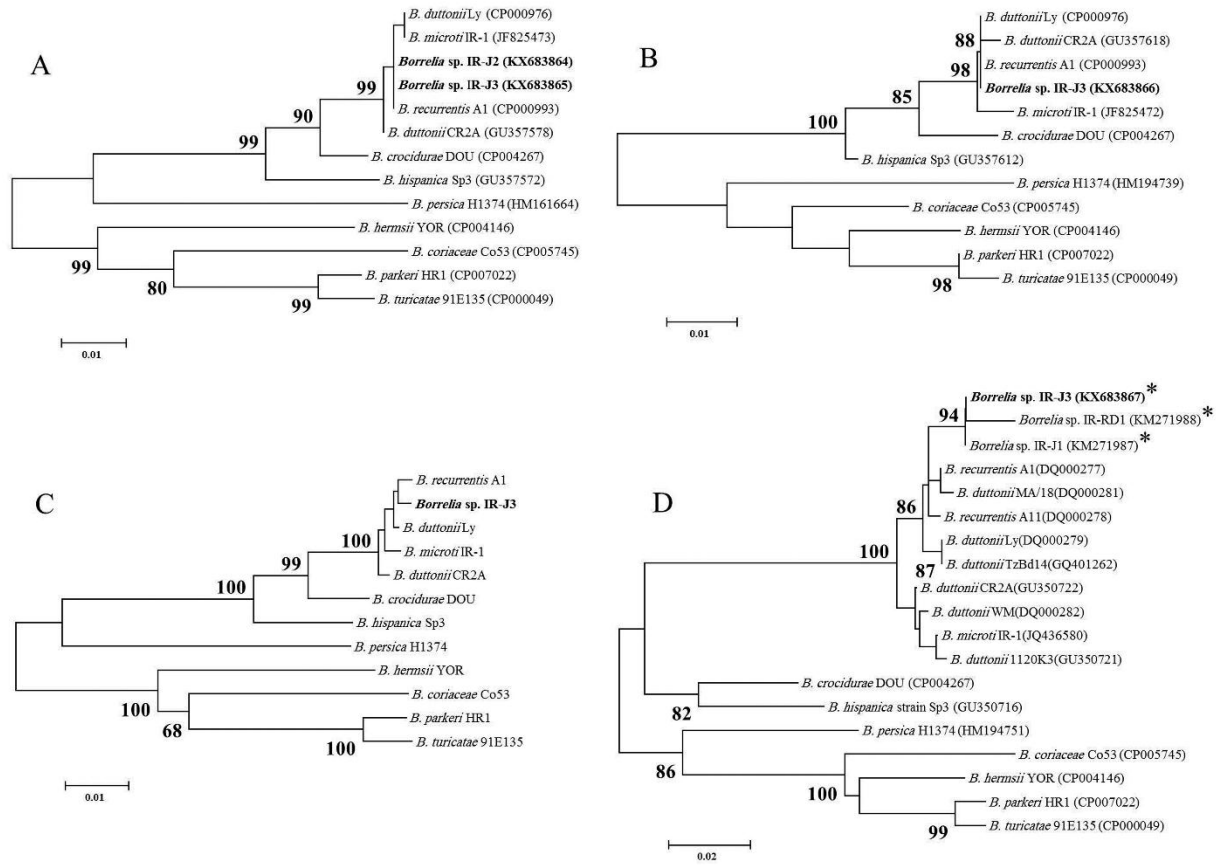


Fig-1

Figr-2



Figr-3

